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Interaction of phlorizin, a potent inhibitor of the Na^+/D-glucose cotransporter, with the NADPH-binding site of mammalian catalases

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Abstract

Phlorizin is a reversible inhibitor of the renal and small intestinal Na^+/D-glucose cotransporter. In an attempt to purify the Na^+/D-glucose cotransporter from a pig kidney brush border membrane fraction, we used an Affi-Gel affinity chromatography column to which 3-aminophlorizin had been coupled. A protein, composed according to crosslinking experiments of at least 3 subunits of molecular weight 60 kDa, was found to bind specifically to the phlorizin column. This protein was subsequently identified as catalase by sequence homology of three of its tryptic fragments to the sequence of several mammalian catalases as well as by its enzymatic activity. Although bovine liver catalase was bound tightly to the affinity matrix, phlorizin had no effect on the ability of the enzyme to degrade H_2O_2. In contrast, the *Aspergillus niger* and *Neurospora crassa* catalases did not bind to the phlorizin column. This difference may be related to the fact that mammalian catalases, but not the fungal catalases, contain an NADPH binding site with a yet unknown function. Interestingly, bovine liver catalase could be eluted with 50 μM NADPH from phlorizin columns. Irradiation in the presence of [^{3}H]4-azidophlorizin allowed photolabeling of bovine liver catalase, which was prevented by the presence of 10 μM NADPH. After digestion of photolabeled catalase with chymotrypsin, a radioactive peptide was detected that was absent in catalase protected with NADPH. Docking simulations suggested that phlorizin can bind to the NADPH binding site with high affinity.

Keywords: affinity chromatography; catalase; NADPH binding site; Na^+/D-glucose cotransporter; phlorizin

The Na^+/D-glucose cotransporter catalyzes the Na^+-dependent uptake of D-glucose into epithelial cells of the renal proximal tubules and small intestine (Silverman, 1991; Burckhardt & Kinne, 1992). The gene encoding the Na^+/D-glucose cotransporter has been cloned from several sources including rabbit small intestine and rabbit kidney proximal tubules (Hediger et al., 1987; Morrison et al., 1991). However, no homogeneous preparation of the Na^+/D-glucose cotransporter protein has so far been obtained. Phlorizin has been shown to be a reversible inhibitor of this transport protein (Diedrich, 1965). In an attempt to purify the Na^+/D-glucose cotransporter from pig kidney cortex, we used affinity chromatography on columns carrying 3-aminophlorizin. A protein, composed of subunits of 60 kDa and suspected by us to be the Na^+/D-glucose cotransporter, was specifically bound to the phlorizin column (Kitlar et al., 1988). However, due to sequence homology of 3 tryptic fragments, we identified this protein as catalase. Phlorizin seems to interact with the NADPH binding site of mammalian catalases. NADPH was reported to bind with high affinity (K_d lower than 10^{-8} M) to mammalian catalases (Kirkman & Gaetani, 1984). The structure of the ligand bound to catalase differs from the extended structure it attains in NADP-dependent dehydrogenases (Melik-Adamyan et al., 1986). Fungal catalases cannot bind NADPH (Melik-Adamyan et al., 1986) and are not retarded by the phlorizin column. The results obtained for the phlorizin binding site of catalase are expected to provide an insight into the yet undetermined phlorizin binding site of the Na^+/D-glucose cotransporter.

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Results

In an attempt to purify the Na^+/D-glucose cotransporter by affinity chromatography on phlorizin columns (3-aminophlorizin

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          156                    167
pig        -D-A-I-L-F-P-S-F-T-H-G-Q-
bovine     -*-*-L-*-*-*-*-*-I-*-S-*-
human      -*-P-*-*-*-*-*-*-I-*-S-*-
rat        -*-*-M-*-*-*-*-*-I-*-S-*-

          170                    182
pig        -N-P-Q-T-H-L-K-D-P-D-M-V-W-
bovine     -*-*-*-*-*-*-*-*-*-*-*-*-*-
human      -*-*-*-*-*-*-*-*-*-*-*-*-*-
rat        -*-*-*-*-*-*-*-*-*-*-*-*-*-

          431                    442
pig        -F-N-S-A-N-E-D-N-V-T-Q-V-
bovine     -*-*-*-*-*-D-*-*-*-*-*-*-
human      -*-*-T-*-*-D-*-*-*-*-*-*-
rat        -*-*-T-*-*-*-*-*-*-*-*-*-
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Fig. 1. Amino acid sequences of 3 tryptic peptides derived from the protein purified by affinity chromatography on phlorizin columns (labeled as pig) and sequence homology to 3 regions of mammalian catalases (bovine liver catalase, Schroeder et al. [1982]; human fibroblast catalase, Korneluk et al. [1984]; rat liver catalase, Furuta et al. [1986]). Asterisks indicate amino acids identical to amino acids in the sequence of the 3 tryptic peptides. Numbers refer to the sequence of bovine liver catalase.

coupled to Affi-Gel 15; Bio-Rad, München), we had isolated from the brush border membrane fraction of pig kidney outer cortex a protein composed of at least 3 subunits of molecular weight 60 kDa according to crosslinking experiments (Kitlar et al., 1988). We purified 3 tryptic peptides from this protein by in situ proteolysis as described under Materials and methods. The 3 peptides were sequenced by automated Edman degradation, and the sequences, presented in Figure 1, were determined. None of the peptides showed homology to the deduced amino acid sequence determined for the rabbit small intestinal and rabbit renal Na^+/D-glucose cotransporter (Hediger et al., 1987; Morrison et al., 1991). A computer search in the Swiss-Prot protein database revealed that the 3 peptides showed homology to 3 different regions of the protein sequence of mammalian catalases (Schroeder et al., 1982; Korneluk et al., 1984; Furuta et al., 1986) as outlined in Figure 1. In addition, the protein purified by affinity chromatography on phlorizin columns exhibited catalase activity. Polyclonal antibodies against this protein reacted with bovine liver catalase in Western blot analysis (Fig. 2A) and antibodies against bovine liver catalase reacted with the protein purified from pig kidney (Fig. 2B). Bovine liver catalase (2 mg) bound almost quantitatively to the phlorizin affinity resin (2 mL) (Fig. 3) and could be eluted from the column with phlorizin (0.2 mM). As described for the binding of the purified protein to phlorizin columns (Kitlar et al., 1988), binding of bovine liver catalase to phlorizin columns was also stronger in the presence of 100 mM NaCl. These data indicated that the protein purified by affinity chromatography on phlorizin columns is identical with pig kidney catalase.

Phlorizin at concentrations up to 0.1 mM had no inhibitory effect on pig kidney and bovine liver catalase activity, suggesting that phlorizin does not interfere with the active site of catalase. Mammalian catalases contain an NADPH binding site distinct from the catalytic site (Kirkman & Gaetani, 1984). Fungal catalases do not contain this binding site (Melik-Adamyan et al., 1986). Because the catalases from *Aspergillus niger* (Sigma, Heidelberg) and *Neurospora crassa* (provided by W.-H. Kunau) did not bind to the phlorizin column, we suspected that phlorizin interacts with the NADPH binding site characteristic for mammalian catalases. This assumption was strengthened by the finding that bovine liver catalase, bound to a phlorizin affinity column, could be eluted quantitatively with 50 μM NADPH (Fig. 3).

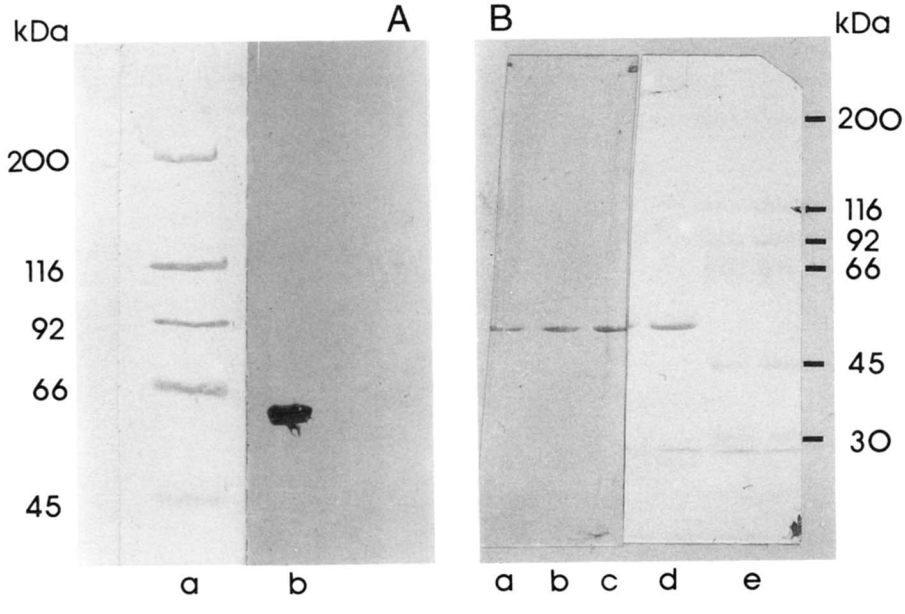

Fig. 2. Western blot analysis (**A**) of 10 μg bovine liver catalase (lane b) by using antibodies directed against the pig kidney protein isolated by affinity chromatography on phlorizin columns, and (**B**) of varying amounts of the purified pig kidney protein (0.5, 1, and 2 μg in lanes a, b, and c, respectively) by using antibodies directed against bovine liver catalase. Lane a in Figure 2A and lane e in Figure 2B contain molecular weight standards, and lane d in Figure 2B contains 5 μg of the purified 60-kDa protein. The proteins in these 3 lanes were stained with Ponceau S after blotting on nitrocellulose.

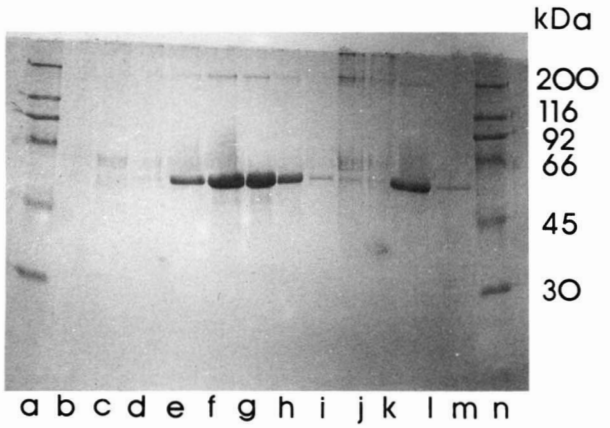

Fig. 3. Detection by SDS-PAGE of bovine liver catalase after loading of 2 mL of a 1-mg/mL bovine liver catalase solution onto a phlorizin column (2 mL resin) and subsequent elution with NADPH. The following samples were loaded on the gel: lanes a and n, molecular weight standards; lane 1, 10 μL of bovine liver catalase before loading onto the phlorizin column; lane m; 100-μL aliquot of the effluent during loading of the phlorizin column; lanes b–d, 100-μL aliquots of the wash fractions after loading the column with bovine liver catalase; lanes e–i, 20-μL aliquots of the fractions collected during elution with 50 μM NADPH; lanes j and k, 20-μL aliquots of the fractions collected during subsequent elution with a 0.1% SDS solution. The fractions collected during the different elution and washing procedures had a volume of 0.7 mL.

We subsequently used [^{3}H]4-azidophlorizin to carry out photolabeling of bovine liver catalase. We found that catalase could be effectively labeled by irradiation for 1 min at 254 nm in the presence of 6 μM [^{3}H]4-azidophlorizin (Fig. 4, lane b). Labeling of bovine liver catalase was abolished in a dose-dependent manner by NADPH over the range of 1–6 μM (Fig. 4, lanes c–e), suggesting that the ligands compete for the binding site. Photolabeling with [^{3}H]4-azidophlorizin of ovalbumin, which unspecifically binds phlorizin, most likely due to hydrophobic interactions, was not affected by the presence of NADPH at concentrations ranging from 1 to 6 μM (Fig. 4, lanes f–h).

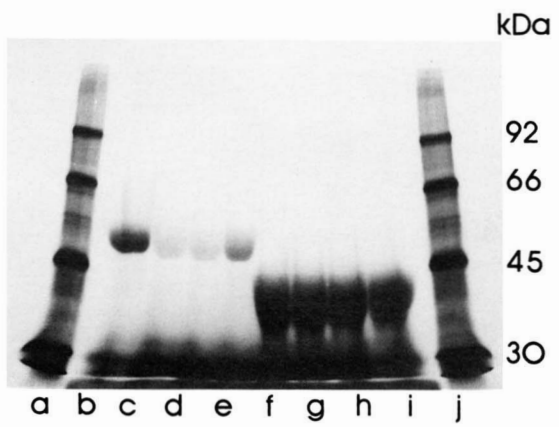

Fig. 4. Fluorography of a 10% SDS-polyacrylamide gel. The following samples were loaded onto the gel: lanes a and j, ^{14}C-labeled molecular weight standards; lane b, 10 μg of bovine liver catalase photolabeled in the presence of 6 μM [^{3}H]4-azidophlorizin; lanes c–e, 10 μg of bovine liver catalase protected against photolabeling by 6, 3, and 1 μM NADPH, respectively; lane f, 20 μg of photolabeled ovalbumin; lanes g–i, 20 μg of ovalbumin; the photolabeling was carried out in the presence of NADPH (6, 3, and 1 μM for lanes g–i, respectively) and in the presence of 6 μM [^{3}H]4-azidophlorizin.

The binding of [^{3}H]4-azidophlorizin to catalase seems to occur at a single site. When photolabeled catalase was digested with chymotrypsin and the resulting peptides were subsequently separated by HPLC on a C-8 reverse-phase column, we observed only a single peak of radioactivity (Fig. 5, panel A1). This peak of radioactivity was absent in catalase preparations where the photolabeling had been prevented by the presence of 10 μM NADPH (Fig. 5, panel B1). However, we were not able to purify the labeled peptide to homogeneity. All of the chromatographic systems employed to fractionate the phlorizin-containing peptide further were found to bind the labeled fragment tightly, hence leading to a drastic loss of the labeled peptide during purification. The feature of phlorizin to bind strongly to different column materials (Diedrich, 1990) is most likely responsible for this effect.

Because the crystal structure of bovine liver catalase has been resolved (Fita & Rossmann, 1985), we were able to carry out docking simulations for the binding of phlorizin to the NADPH binding site as described under Materials and methods. These simulations suggested a possible binding of phlorizin to the NADPH binding site of catalase with a binding energy of -111 kJ/mol, which is comparable to the value of -120 kJ/mol calculated for the binding of NADPH to this site.

Discussion

We have previously reported the purification of a protein composed of at least 3 identical 60-kDa subunits from a brush border membrane fraction of the pig kidney outer cortex. We assumed this protein to be the Na^+/D-glucose cotransporter, because it was purified by affinity chromatography on phlorizin columns (Kitlar et al., 1988) and because phlorizin is a potent inhibitor of the Na^+/D-glucose cotransporter. However, the brush border membrane fraction, which was obtained by following an established preparation method (Lin et al., 1981), appeared to be contaminated with peroxisomes, because we identified this protein as catalase. The identification was based on: (1) the homology (greater than 75%) of 3 tryptic fragments of the purified protein to 3 different regions of mammalian catalases; (2) the catalytic activity exhibited by the purified protein; (3) the binding of bovine liver catalase to phlorizin columns; and (4) immunoblotting experiments using polyclonal antibodies against the protein purified by affinity chromatography, which also recognized bovine liver catalase.

Phlorizin had no effect on the activity of pig kidney and bovine liver catalase. Because the catalases of *A. niger* and *N. crassa*, which are assumed not to possess the NADPH binding site described for mammalian catalases (Kirkman & Gaetani, 1984), did not bind to a phlorizin column, we suspected that phlorizin interacts with the NADPH binding site. This assumption was supported by the finding that bovine liver catalase, bound to phlorizin columns, could be eluted with low concentrations of NADPH. In addition, photolabeling of bovine liver catalase with [^{3}H]4-azidophlorizin was inhibited by the presence of micromolar concentrations of NADPH. A ratio of the corresponding Michaelis constants K_m(phlorizin)/K_m(NADPH) = 3 was obtained from the calculated binding energies of phlorizin and NADPH to the NADPH binding site of bovine liver catalase.

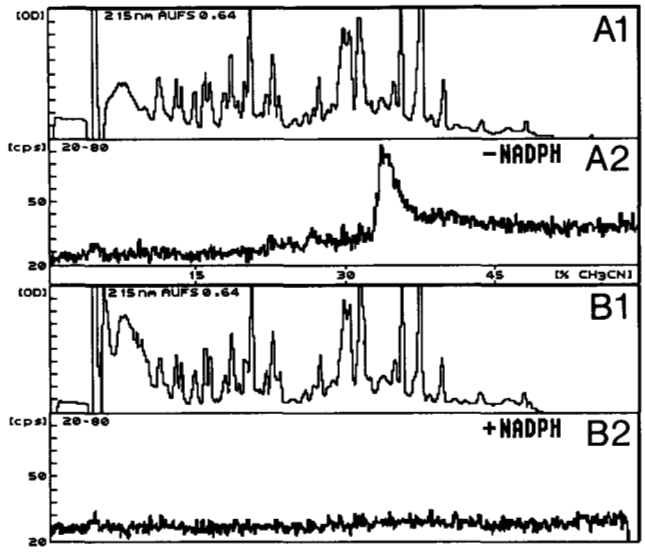

Fig. 5. Separation by HPLC of chymotryptic fragments derived from (**A**) photolabeled and (**B**) NADPH-protected bovine liver catalase. Panels A1 and B1 show the UV-absorption measured at a wavelength of 215 nm. Panels A2 and B2 show the distribution of radioactivity determined in the effluent as described in the Materials and methods.

The binding of catalase to phlorizin not only provides a quick and efficient method to purify this enzyme from mammalian sources by affinity chromatography, but it will also be helpful in determining the function of NADPH bound to catalase. The NADPH found in bovine liver catalase has a folded structure that is different from the extended form of NADPH observed in NADP(H)-dependent dehydrogenases (Melik-Adamyan et al., 1986). It has been suggested that NADPH bound to catalase serves to protect the enzyme against oxidation by H_2O_2, but additional functions of NADPH have been implicated (Kirkman et al., 1987). Affinity chromatography on phlorizin columns might also be used for a screening to detect other NADPH binding proteins containing NADPH with a folded structure. According to the suggested function of NADPH bound to catalase, such enzymes are likely to be found in peroxisomes. Like catalase, they should be retarded by phlorizin columns and be eluted with NADPH.

The knowledge of the phlorizin binding site of catalases might also be helpful in determining the phlorizin binding site of the Na^+/D-glucose cotransporter. Although there is no obvious sequence homology between the Na^+/D-glucose cotransporter and the amino acid strands forming the NADPH binding site of catalase, it is possible that the respective phlorizin binding sites resemble each other on the level of secondary and tertiary structure.

Materials and methods

Protein and peptide purification

A protein, composed of 60-kDa subunits and later on identified as the enzyme catalase, was purified on a phlorizin affinity column and by reverse-phase HPLC (Kitlar et al., 1988). Because the protein preparation obtained after the 2 purification steps was still found to be contaminated with protease activity (which did not allow the isolation of defined tryptic fragments), we carried out in situ proteolysis (Aebersold et al., 1987). For this purpose, 300 μg of the purified protein was loaded onto a preparative SDS polyacrylamide gel and blotted onto nitrocellulose after electrophoresis. Proteins were visualized by staining with Ponceau S (Serva, Heidelberg), the 60-kDa protein band was cut out, and the protein was subsequently digested with trypsin (Boehringer, Mannheim) as described by Aebersold et al. (1987). Peptides were purified on a C-8 reverse-phase column by applying a 90-min linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid, pH 2. We chose 3 peptides for sequencing that appeared to be homogeneous according to thin-layer chromatography on HPTLC plates (Nano Sil C_{18-20}, Macherey & Nagel, Düren). Protein sequencing was carried out by automated Edman degradation on a gas phase sequencer according to Hewick et al. (1981).

Enzyme assay

Catalase activity was determined photometrically by measuring the decrease of the H_2O_2 concentration at a wavelength of 254 nm as described by Beers and Sizer (1951).

Gel electrophoresis, fluorography, and immunoblotting

SDS-PAGE was performed according to Laemmli (1970). Gels were treated with Amplify (Amersham, Braunschweig) before they were exposed for fluorography. Western blot analysis was carried out as described by Kitlar et al. (1988). Polyclonal antibodies against the protein purified by affinity chromatography on phlorizin columns and against bovine liver catalase were obtained as previously described (Kitlar et al., 1988).

Synthesis of [^{3}H]4-azidophlorizin and photolabeling experiments

[^{3}H]4-aminophlorizin was synthesized from brominated 4-aminophlorizin by a tritium exchange reaction with a specific activity of 6.7×10^{11} Bq/mmol and was subsequently converted to [^{3}H]4-azidophlorizin by following the method described by Diedrich (1990). For photolabeling of bovine liver catalase (Serva, Heidelberg), a sample containing 10 mM MES buffer, pH 6.2, 100 mM NaCl, 20 μM protein, and 6 μM [^{3}H]4-azidophlorizin was irradiated for 1 min at a wavelength of 254 nm (13 watts) from a distance of 2 cm. Protection against photolabeling of catalase was carried out by using varying concentrations of NADPH (1, 3, and 6 μM). The reaction mixtures were loaded onto Sephadex G-10 columns (Pharmacia, Freiburg; 0.5×10 cm) to remove unbound [^{3}H]4-azidophlorizin from the labeled proteins. Peptides derived from photolabeled and protected catalase (100 μg each) were obtained by digestion with 5 μg chymotrypsin (Boehringer, Mannheim) for 4 h. The separation of peptides was carried out on a C-8 reverse-phase column employing the same conditions as described for the purification of tryptic fragments from the protein purified by affinity chromatography (see above). Radioactivity of the effluent was detected on line by using a flow-through radioactivity monitor equipped with a detection cell containing a solid-phase scintillator (Berthold, Wildbad).

Docking simulations

Monte-Carlo-type docking simulations were carried out using the CONDOR program of Hoechst corporate research, which was run on a VAX 8650 computer. An Evans and Sutherland PS300 graphics system together with modeling software of Hoechst was used for modeling work. The calculations were based on the AMBER 3.0 force field (Weiner et al., 1984). Binding energies were determined from the force field energies of catalase, NADPH, phlorizin, and the complexes of catalase with NADPH and phlorizin in the energetically most favorable conformations found by the docking simulations. Solvation energies were not taken into account because solubility in water is similar for NADPH and phlorizin. There is, however, an important entropy contribution caused by restriction of freely rotatable bonds of the ligands on complex formation. NADPH has 12 such bonds, whereas phlorizin has only 6. We assumed an entropical loss of free energy $T \times \Delta S = 3$ kJ/mol per bond at 300 K (Williams et al., 1993).

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